~~HYBRID TOXIN~~ GENES ENCODING HYBRID BACILLUS THURINGIENSIS TOXINS

This application is a continuation-in-part of application serial no. 08/602,737, filed February 21, 1996, which is a 371 of international application no. PCT/EP94/02909, filed September 1, 1994. Both of the aforementioned applications are incorporated herein by reference.

FIELD OF THE INVENTION

The present invention relates to hybrid toxin fragments, and toxins comprising them, derived from *Bacillus thuringiensis* insecticidal crystal proteins.

BACKGROUND OF THE INVENTION

Bacillus thuringiensis (hereinafter B.t.) is capable of producing proteins that accumulate intra-cellularly as crystals. These crystal proteins are toxic to a number of insect larvae. Based on sequence homology and insecticidal specificity, crystal proteins have been categorized into different classes. Best studied are the CryI class of proteins, which are produced as 140 kDa protoxins and are active towards lepidopterans.

To some extent, the mode of action of crystal proteins has been elucidated. After oral uptake, the crystals dissolve in the alkaline environment of the larval midgut. The solubilized proteins are subsequently processed by midgut proteinases to a proteinase-resistant toxic fragment of about 65kDa, which binds to receptors on epithelial cells of the insect midgut and penetrates the cell membrane. This eventually leads to bursting of the cells and death of the larvae.

The activity spectrum of a particular crystal protein is to a large extent determined by the occurrence of receptors on the midgut epithelial cells of susceptible insects. The activity spectrum is co-determined by the efficiency of solubilization of the crystal protein and its proteolytic activation *in vivo*.

The importance of the binding of the crystal protein to midgut epithelial receptors is further demonstrated where insects have developed resistance to one of the crystal proteins, such that the binding of crystal proteins to midgut epithelial cells in resistant insects is significantly reduced.

Toxic fragments of crystal proteins are thought to be composed of three distinct structural domains. Domain I, the most N-terminal domain, consists of 7 α-helices. Domain II comprises 3 ß-sheets. Domain III, the most C-terminal domain, folds into a ß-sandwich. If projected on CryI sequences, domain I runs from about amino acid residues 28 to 260, domain II from about 260 to 460, and domain III from about 460 to 600.

DESCRIPTION OF THE INVENTION

The present invention concerns hybrid crystal proteins particularly, though not exclusively, involving CryIC together with CryIE, CryIA, or CryIG. The nucleotide sequence of the CryIC gene from B.t. sub. sp. *entomocidus* 60.5 is given in SEQ ID NO:1, and the corresponding amino acid sequence of the protein encoded by said nucleotide sequence is given in SEQ ID NO:2. The nucleotide sequence of the CryIE gene from B.t. sub. sp. *kenyae* 4FI is given in SEQ ID NO:3, and the corresponding amino acid sequence of the protein encoded by said nucleotide sequence is given in SEQ ID NO:4. The nucleotide sequence of a B.t. CryIG gene is given in SEQ ID NO:9, and the corresponding amino acid sequence of the protein encoded by said nucleotide sequence is given in SEQ ID NO:10. These proteins are toxic to lepidopterans, but within this order of insects, each protein has different specificity. CryIC, for example, is particularly active against *S. exigua* and *M. brassicae*.

According to the present invention, there is provided an isolated B.t. hybrid toxin fragment comprising at its C-terminus domain III of a first Cry protein, or a part of said domain or a protein substantially similar to said domain; and comprising at its N-terminus the N-terminal region of a second Cry protein, or a part of said region or a protein substantially similar to said region. For example, a preferred B.t. hybrid toxin fragment according to the present invention comprises at its C-terminus domain III of a first Cry protein and comprises at its N-terminus domains I and II of a second Cry protein. A preferred fragment is one that does not bind to the CryIC binding site in an insect gut when it comprises at its C-terminus domain III of CryIC, or a part of said domain or a protein substantially similar to said domain; or one that does not bind to a CryIA binding site when it comprises at its C-terminus domain III of CryIA, or a part of said domain or a protein substantially similar to said domain.

In the context of the present invention, "substantially similar" means a pure protein having an amino acid sequence that is at least 75% similar to the sequence of a protein according to the invention. It is preferred that the degree of similarity is at least 85%, more preferred that the degree of similarity is at least 90%, and still more preferred that the degree of similarity is at least 95%. In the context of the present invention, two amino acid sequences with at least 75%, 85%, 90%, or 95% similarity to each other have at least 75%, 85%, 90%, or 95% identical or conservatively replaced amino acid residues in a like position when aligned optimally allowing for up to 6 gaps, with the proviso that, with respect to the gaps, a total not more than 15 amino acid residues are affected. For the purpose of the present invention, conservative replacements may be made between amino acids within the following groups:

(i) Serine and Threonine;

(ii) Glutamic acid and Aspartic acid;

(iii) Arginine and Lysine;

(iv) Asparagine and Glutamine;

(v) Isoleucine, Leucine, Valine, and Methionine;

(vi) Phenylalanine, Tyrosine, and Tryptophan; and

(vii) Alanine and Glycine,

with the proviso that in SEQ ID NO:6, Ser and Tyr are conservative replacements at position 620, and Ala and Glu are conservative replacements at position 618; and that in SEQ ID NO:8, Ser and Tyr are conservative replacements at position 627, and Ala and Glu are conservative replacements at position 625.

In the context of the present invention, "part" of a protein means a peptide comprised by said protein and having at least 80% of the consecutive sequence thereof.

In the context of the present invention, "binding site" means a site on a molecule wherein the binding between site and toxin is reversible such that the *Ka* between site and toxin is in the order of at least $10^4 dm^3 mole^{-1}$.

The toxin fragment may comprise at its N-terminus the N-terminal region of any insecticidal protein from B.t. being commonly known as "Cry" or "Cyt", including: CryIA(a),

CryIA(b) CryIA(c), CryIB, CryIC, CryID, CryIE, CryIF, CryIG, CryIH, CryIIA, CryIIB, CryIIC, CryIIIA, CryIIIB, CryIIIB(b), CryIVA, CryIVB, CryIVC, CryIVD, CYTA, CryX1(IIIC), CryX2(IIID), CryX3, CryV, and CryX4, or a part of said region or a protein substantially similar to said region. The toxin fragment may comprise at its C-terminus domain III of CryIC, or a part of said domain or a protein substantially similar to said domain.

Thus, the fragment may comprise domain II of CryIE, CryIB, CryID, CryIA, or CryIG, or a part of said domain II or a protein substantially similar to said domain II, and domain III of CryIC or a part of said domain III or a protein substantially similar to said domain III. It is particularly preferred that the fragment comprises domains I and II of CryIE, CryIB, CryID, CryIA, or CryIG, or a part thereof or a protein substantially similar to said domains I and II, and domain III of CryIC or a part thereof or a protein substantially similar to said domain III.

It is most preferred that the toxin fragment comprises a region at its C-terminus comprising the sequence from amino acid position 454 to position 602 of CryIC, or a sequence substantially similar to said sequence. The fragment may comprise a region at its C-terminus comprising the sequence from amino acid position 478 to 602 of Cry IC, or a sequence substantially similar to said sequence, with the proviso that if the sequence comprising amino acids 478 to 602 of CryIC is fused directly to the C-terminus of domain II of CryIA, CryIB, CryID, CryIE, or CryIG, then the folding of the fusion product is satisfactory to yield an insecticidal component of the fragment. The routineer in the art will recognize that it may be necessary to add a peptide region to the C-terminus of domain II that spaces the C-terminal region of CryIC apart, thus enabling it to fold in such a way as to exhibit insecticidal activity.

It is most particularly preferred that the toxin fragment according to the invention comprises one of the following:

i) an amino acid sequence from about amino acid 1 to about amino acid 620 in SEQ ID NO:6, or an amino acid sequence from about amino acid 1 to about amino acid 620 in SEQ ID NO:6, wherein with respect to said sequence, at least one of the following alterations is present:

Ile at position 609 is replaced with Leu,

Ala at position 618 is replaced with Glu,

Ser at position 620 is replaced with Tyr;

ii) an amino acid sequence from about amino acid 1 to about amino acid 627 in SEQ ID NO:8, or an amino acid sequence from about amino acid 1 to about amino acid 627 in SEQ ID NO:8, wherein with respect to said sequence, at least one of the following alterations is present:

Ile at position 616 is replaced with Leu,

Ala at position 625 is replaced with Glu,

Ser at position 627 is replaced with Tyr; and

iii) an amino acid sequence from about amino acid 1 to about amino acid 602 in SEQ ID NO:12.

Whatever amino acid alterations are permitted, however, one or more of the following residues indicated sequence-wise with respect to the CryIC sequence is invariable: Phe (501), Val (478), Trp (479), and Thr (486).

The invention also includes a hybrid toxin comprising the above disclosed fragment or a toxin at least 85% similar to such a hybrid toxin, which has substantially similar insecticidal activity or receptor binding properties.

The invention still further includes pure proteins that are at least 90% similar to the toxin fragments or hybrid toxins according to the invention.

The invention still further includes recombinant DNA comprising a sequence encoding a protein comprising an amino acid sequence of one of the above-disclosed toxins or fragments thereof. The invention still further includes recombinant DNA comprising the sequence from about nucleotide 1 to about nucleotide 1860 given in SEQ ID NO:5, or DNA similar thereto encoding a substantially similar protein; or recombinant DNA comprising the sequence from about nucleotide 1 to about nucleotide 1881 in SEQ ID NO:7, or DNA similar thereto encoding a substantially similar protein; or recombinant DNA comprising the sequence from about nucleotide 1 to about nucleotide 1806 in SEQ ID NO:11, or DNA similar thereto encoding a substantially similar protein.

In the context of the present invention, "similar DNA" means a test sequence that is capable of hybridizing to the inventive recombinant sequence. When the test and inventive sequences are

double stranded, the nucleic acid constituting the test sequence preferably has a TM within 20°C of that of the inventive sequence. In the case that the test and inventive sequences are mixed together and denatured simultaneously, the TM values of the sequences are preferably within 10°C of each other. More preferably, the hybridization is performed under stringent conditions, with either the test or inventive DNA preferably being supported. Thus, either a denatured test or inventive sequence is preferably first bound to a support and hybridization is effected for a specified period of time at a temperature of between 50°C and 70°C in double strength citrate buffered saline containing 0.1% SDS, followed by rinsing of the support at the same temperature but with a buffer having a reduced SC concentration. Depending upon the degree of stringency required, and thus the degree of similarity of the sequences, such reduced concentration buffers are typically single strength SC containing 0.1% SDS, half strength SC containing 0.1% SDS and one tenth strength SC containing 0.1% SDS. Sequences having the greatest degree of similarity are those the hybridization of which is least affected by washing in buffers of reduced concentration. It is most preferred that the test and inventive sequences are so similar that the hybridization between them is substantially unaffected by washing or incubation in one tenth strength sodium citrate buffer containing 0.1% SDS. Typical stringent conditions are as follows: hybridization at 7% sodium dodecyl sulfate (SDS), 0.5 M $NaPO_4$ pH 7.0, 1 mM EDTA at 50°C; wash with 2X SSC, 1% SDS, at 50°C.

The recombinant DNA may further encode a protein having herbicide resistance, plant growth-promoting, anti-fungal, anti bacterial, anti-viral, and/or anti-nematode properties. In the case that the DNA is to be introduced into a heterologous organism, it may be modified to remove known mRNA instability motifs (such as AT rich regions) and polyadenylation signals, and/or codons that are preferred by the organism into which the recombinant DNA is to be inserted may be used so that expression of the thus modified DNA in the organism yields substantially similar protein to that obtained by expression of the unmodified recombinant DNA in the organism in which the protein components of the hybrid toxin or toxin fragments are endogenous.

The invention still further includes a DNA sequence complementary to one that hybridizes under stringent conditions with the recombinant DNA according to the invention.

Also included in the present invention are the following: a vector containing such a recombinant (or complementary thereto) DNA sequence; a plant or microorganism that includes and enables expression of such DNA; plants transformed with such DNA; the progeny of such plants that contain the DNA stably incorporated and hereditable in a Mendelian manner; and/or the seeds of such plants and such progeny.

The invention still further includes protein derived from expression of the recombinant DNA of the invention, and insecticidal protein produced by expression of the recombinant DNA within plants transformed therewith.

The invention still further includes the following: an insecticidal composition containing one or more of the toxin fragments or toxins comprising them according to the invention; a process for combating insects that comprises exposing them to such fragments or toxins or compositions; and an extraction process for obtaining insecticidal proteins from organic material containing them, comprising submitting the material to maceration and solvent extraction.

DESCRIPTION OF THE FIGURES

Figure 1 shows the generation of hybrid crystal protein genes via *in vivo* recombination. Tandem plasmids (pBD560 and pBD 650) carrying two truncated crystal protein genes in direct repeat orientation are constructed. The 5' located gene (open bar) lacks the protoxin encoding region (solid bar) and of the 3' located gene (dashed bar) part of the domain I encoding region is deleted. *In vivo* recombination between homologous regions (domain II and III) occurs in *rec*A + strain JM101. Selection against non-recombinants by digestion with *Not*I and *Bam*HI and subsequent transformation results in sets of plasmids encoding hybrid crystal proteins.

Figure 2 shows the alignment of amino acid residues 420 to 630 of CryIE and CryIC. The border between domain II and III is indicated. Only amino acid residues of CryIC that differ from CryIE are depicted; identical residues are indicated by dots. The crossover positions (G27, H13, H7, H8, H17, and H21) in the CryIE/CryIC hybrid toxin fragments according to the invention are indicated on the Figure.

Figure 3 shows the alignment of amino acid residues 420 to 630 of CryIE and CryIC. The border between domain II and III is indicated. Only amino acid residues of CryIC that differ from CryIE are depicted; identical residues are indicated by dots. The crossover positions (F59, F71, F26, and E7) in the CryIC/CryIE hybrid toxin fragments are indicated on the Figure.

Figure 4 shows the results of heterologous competition experiments. Biotinylated CryIC (panel A) and G27 (panel B) are incubated with *S. exigua* BBMV vesicles in the absence (lanes a) or presence of an excess of unlabelled protein as indicated. After the incubation, the vesicles are washed, loaded on a SDS-polyacrylamide gel and blotted to a nitrocellulose membrane. Biotinylated crystal proteins, re-isolated with the vesicles, are visualized using streptavidin-peroxidase conjugate and are indicated on the Figure with an arrow head.

Figure 5 shows the plasmid map of pSB456, which encodes the G27 hybrid toxin fragment and is used to transform the crystal toxin minus strain B.t. 51.

Figure 6A shows the alignment of the *cry1G* and *cry1C* genes with the crossover points of the *cry1G/cry1C* hybrids. The position relative to the first nucleotide of the start codon of *cry1G* is shown.

Figure 6B shows the alignment of the encoded Cry1G and Cry1C proteins with the crossover points of the Cry1G/Cry1C hybrids. The approximate position of the domain II-III border is indicated by #. The position relative to the initiation codon of Cry1G is also indicated.

Figure 7 shows the results of assays measuring the toxicity of Cry1G/Cry1C hybrid toxins towards *Spodoptera exigua*.

DESCRIPTION OF THE SEQUENCES IN THE SEQUENCE LISTING

SEQ ID NO:1 shows the nucleotide sequence of the CryIC gene from B.t. sub. sp. *entomocidus* 60.5.

SEQ ID NO:2 shows the amino acid sequence of the protein encoded by the CryIC gene shown in SEQ ID NO:1.

SEQ ID NO:3 shows the nucleotide sequence of the CryIE gene from B.t. sub. sp. *kenyae* 4FI.

SEQ ID NO:4 shows the amino acid sequence of the protein encoded by the CryIE gene shown in SEQ ID NO:3.

SEQ ID NO:5 shows the nucleotide sequence encoding a preferred CryIE/CryIC B.t. hybrid toxin fragment according to the invention.

SEQ ID NO:6 shows the amino acid sequence of the protein encoded by the nucleotide sequence shown in SEQ ID NO:5.

SEQ ID NO:7 shows the nucleotide sequence of a CryIA/CryIC hybrid toxin fragment according to the invention.

SEQ ID NO:8 shows the amino acid sequence of the protein encoded by the nucleotide sequence depicted in SEQ ID NO:7.

SEQ ID NO:9 shows the nucleotide sequence of a B.t. CryIG gene.

SEQ ID NO:10 shows the amino acid sequence of the protein encoded by the CryIG gene shown in SEQ ID NO:9.

SEQ ID NO:11 shows the nucleotide sequence encoding a preferred CryIG/CryIC B.t. hybrid toxin fragment (hybrid HK28-24) according to the invention.

SEQ ID NO:12 shows the amino acid sequence of the protein encoded by the nucleotide sequence shown in SEQ ID NO:~~12~~ 11.

SEQ ID NOs:13-15 are oligonucleotides.

The invention will be further apparent from the following non-limiting Examples, which describe the production of B.t. hybrid toxin fragments according to the invention, taken in conjunction with the associated Figures and Sequence Listing.

EXAMPLES

Production Of Plasmids Encoding Hybrid Toxin Fragments

In the production of plasmids carrying the CryIC or CryIE genes, *Escherichia coli* XLI-blue (Stratagene Inc.) is used as plasmid host except in cases were JM101 is used as *rec*A+ background. A vector for the expression of crystal proteins in *E. coli* is derived from pKK233-2 (Pharmacia LKB Biotechnology). The size of pKK233-2 is reduced by deleting an *Eco*RI-*Pvu*II fragment carrying the gene encoding tetracycline resistance. Subsequently a 6 bp *Xho*I linker is ligated into the *Hind*III site resulting in pBD10. Plasmid BK+ is created by insertion of a *Bgl*II linker in the *Sac*I site of Bluescript SK+ (Stratagene Inc.). The polylinker of BK+ from *Bgl*II to *Xho*I is introduced between the *Nco*I-*Xho*I site in pBD10. The resulting expression vector pBD11 contains the highly expressed *trc* promoter, the *lac*Z ribosome binding site and ATG initiation codon. The initiation codon overlaps with a *Nco*I site and is followed by the polylinker to facilitate insertions into the vector. Transcription is terminated by the *rrn*B transcription terminator.

The cloning of the *cryIC* and *cryIE* genes from B.t. sub. sp. *entomocidus* 60.5 and *kenya* 4F1 respectively is as described previously (Honée *et al.*, 1990 (Appl. Environ. Microbiol. 56, pp. 823-825); Visser *et al.*, 1990 (J. Bacteriol. 172, pp. 6783-6788)). For cloning purposes, an *NcoI* site overlapping with the start codon of *cryIC* is created by *in vitro* mutagenesis. A *Bgl*II site is created directly downstream of the translation termination codon of cryIC by site directed mutagenesis, resulting in the sequence ATAAGATCTGTT (SEQ ID NO:13 - stop-codon underlined). The *Nco*I-*Bgl*II fragment containing the *cryIC* coding region is ligated into pBD11, resulting in CryIC expression plasmid pBD150. pBD155 is a derivative of pBD150, in which the polylinker sequences 3' of *cryIC* are deleted.

A *Dra*I fragment from pEM14 (Visser *et al.*, 1990) containing the complete *cry*IE gene is cloned in the *Eco*RV site of SK+, resulting in plasmid pEM15. Subsequently, an *Nco*I site is introduced by site directed mutagenesis at the start codon of the gene, and *cryIE* is transferred as an *Nco*I-*Xho*I fragment to pBD11, resulting in CryIE expression plasmid pBD160.

Plasmids carrying only toxic fragment-encoding regions of the *cryI* genes are constructed. *Bgl*II linkers are ligated to *Xmn*I sites present at bp position 1835 of *cryIC*, and to the *Hgi*AI site at position 1839 of *cryIE*. Subsequently, *Nco*I-*Bgl*II fragments containing the *cryIC* (1835 bp) and *cryIE* (1839 bp) toxic fragment-encoding regions are ligated into pBD11, resulting in pBD151 and pBD161 respectively as described below.

Tandem plasmids used for the generation of *cryIC-cryIE* hybrid genes are constructed as follows: *BamH*I linkers are ligated to pBD160 digested with *Hpa*I. This DNA is incubated with *BamH*I and *Xho*I and the truncated *cryIE* gene running from bp 704 is ligated into pBD151 resulting in pBD560. To construct a tandem plasmid for the generation of *cryIE-cryIC* hybrids, pBD155 is digested with *Nsi*I and *Xho*I. The fragment carrying the truncated *cryIC* gene, running from bp 266, is ligated into *PstI/Xho*I digested pBD161, resulting in plasmid pBD650. Due to polylinker sequences, unique *Not*I and *Bam*H1 restriction sites are present between the truncated *cryI* genes present in the tandem plasmids pBD560 and pBD650.

DNA Manipulations And Construction Of Hybrid Toxins

All recombinant DNA techniques are as described by Sambrook *et al.* 1989 (in "Molecular Cloning, A Laboratory Manual: Cold Spring Harbour Press, Cold Spring Harbour). DNA sequencing is performed by the dideoxytriphosphate method with fluorescent dyes attached to the dideoxynucleotides. Analysis is automated by using an Applied Biosystems 370A nucleotide sequence analyzer.

The homology present between *cryI* genes permits intramolecular recombination *in vivo*. Two tandem plasmids are created, each carrying two truncated crystal protein genes overlapping only in domains II and III. Therefore, recombination occurs only in regions encoding domains II and III. In-frame recombinations, which can be selected for by restriction enzyme digestion, generate plasmids that express full size 140 kDa hybrid protoxins. To generate *in vivo* recombinants, a tandem plasmid (either pBD560 or pBD650; Figure 2) is transferred to JM101. 5 mg of DNA is isolated from independently generated recombinants and is digested with *Not*I and *Bam*HI cutting between the two truncated *cryI* genes to select against non-recombinants, and the

DNA is transformed to *E. coli* XL1-blue. 5 single colonies are grown and protein patterns and plasmid content are analyzed.

CryIC/CryIE and CryIE/CryIC hybrid toxins are generated using the tandem plasmids pBD560 and pBD650 respectively, which are allowed to recombine in a *recA*+ background. DNA is isolated, digested, and transferred to *recA*- strain as described above.

100 colonies of 20 independent experiments are analyzed on SDS-PAGE. 85% of these clones produce a 140 kDa protein indicating in frame recombinations between *cryIC* and *cryIE*, and *cryIE* and *cryIC*, respectively. In *E. coli*, CryI proteins are produced as crystals that can be solubilized *in vitro* at high pH. Approximately 15% of hybrid toxins produced as above are solubilized at high pH. The recombinants producing soluble hybrid toxins are first classified using restriction enzymes. Subsequently, for each class, the crossover point of selected hybrids is determined by DNA sequence analysis. All crossovers resulting in soluble hybrid toxins occur in or very close to domain III.

Protein Purification And Analysis

Crystal proteins are isolated essentially as described by Convents *et al.* (J. Biol. Chem. 265, pp. 1369-1375; Eur. J. Biochem., 195, pp. 631-635). Briefly, recombinant *E. coli* are grown at 30°C in 250 ml TB medium to an OD_{660} of 10-15. Crystals isolated from the *E.coli* lysate are solubilized during incubation for 2 hours in 20mM Na_2CO_3, 10 mM dithiothreitol, 100 mM NaCl, pH10, at 37°C. The pH of the solution is lowered to 8 with Tris-HCl and incubated with trypsin. The toxin solution is dialysed against 20 mM Tris-HCl, 100 mM, NaCl pH9. Subsequently, the toxic fragment is purified on a Mono Q 5/5 column connected to a fast-protein liquid chromatography (FPLC) system (Pharmacia LKB Biotechnology). Proteins are separated by 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoreses.

Biochemical Analysis And Isolation Of 65 kDa Toxic Fragments

Isolated crystals of purified CryIC, CryIE, and the hybrid proteins are solubilized at high pH and incubated with trypsin. Like CryIC and CryIE, all soluble hybrid toxins with crossovers in domain III are converted to stable 65 kDa fragments. The 65 kDa fragments can be purified using

anion exchange chromatography under similar conditions as the parental proteins. Hybrids F59 and F71, which have crossovers in domain II, are completely degraded by trypsin. Apparently, although these hybrids do not precipitate as insoluble aggregates, trypsin cleavage sites buried in the parental proteins may become exposed to trypsin. Because of this phenomenon, no 65 kDa fragments are isolated from F59 and F71.

Table 1 shows the constitution of 5 CryIE/CryIC hybrid toxins: (G27, H8, H17, H13, H7, and H21) and 4 CryIC/CryIE hybrid toxins (F59, F71, F26, and E7) with reference to the CryIC and CryIE proteins from which they are derived. The amino acid sequences of the CryIE/CryIC toxins comprising the toxic fragments of the present invention run to amino acid 1189 of the CryIC parent protein. The amino acid sequences of the CryIC/CryIE hybrid toxins run to amino acid 1171 of the CryIE parent protein. Table 1 also shows the relative insecticidal effectiveness of these various hybrid toxins with respect to the CryIC and CryIE proteins.

TABLE 1

Toxin	aa IE	aa IC	*M. sexta*	*S. exigua*	*M. brassicae*
IC	0	28-627	++	++	++
IE	29-612	0	++	-	-
G27	1-474	478-627	++	++(+)	+(+)
H8	1-497	501-627	++	-	-
H17	1-529	533-627	++	-	-
H7	1-577	588-627	-	-	-
H21	1-605	621-627			
F59	421-612	1-423	-	-	-
F71	428-612	1-430	-	-	-
F26	455-612 (1171)	1-458	++	-	-
E7	588-612 (1171)	1-602	++	++	++

Table 1. Constitution and toxicity of hybrid toxins with respect to the parent proteins. Most bioassays were performed with purified toxin fragments. In case of CryIC these run from about aa 28 to about aa 627, and in case of CryIE till 612. The length of complete protoxins is indicated between brackets.

Insect Toxicity Assays And Insecticidal Activity of *cryIC/cryIE* Hybrid Gene Products

Bacterial cultures are concentrated to OD_{660} 6.0, and 100 ml are spotted on 2 cm^2 of artificial diet in a 24-well tissue culture plate. Alternatively, diluted samples of purified toxins are applied to the diet. Second instar larvae of either *S. exigua*, *M. brassicae,* or *M. sexta* are fed on this diet (16 per sample dilution) for 5 days, after which the larval weight is scored. The relative growth (EC50, the concentration giving 50% growth reduction) is determined by calculating the ratio between the mean weight of larvae grown on diet supplemented with toxin and the mean weight of control larvae grown on a diet without toxin. *M. sexta* egg layers are supplied by Carolina Biological Supply Company, North Carolina, USA.

The toxic fragments encoded by the hybrid gene products are tested for activity towards three different insect species as described above. *M. sexta* is susceptible to both CryIC and CryIE. As may be anticipated from their sensitivity to trypsin, hybrids F59 and F71 are not active against this insect (Table 1). Although H7 is converted by trypsin to stable 65 kDa proteins, it is not toxic to *M. sexta*. All of the other hybrids given in Table 1 are toxic and are apparently in the native, biologically active conformation.

The 65 kDa fragment of CryIC is highly toxic towards *S. exigua* and *M. brassicae,* whereas CryIE is not. G27 (Table 1; Figure 2), a CryIE-CryIC hybrid with a crossover at the junction of domain II and III is active towards both insects. This demonstrates that domain III of CryIC confers full activity towards *S. exigua* and *M. brassicae*. Hybrid H8, which differs in only three amino acid residues (see Figure 3) from G27, although active against *M. sexta*, is not active against *S. exigua* and *M. brassicae*.

F26 (Table 1; Figure 3), the reciprocal hybrid of G27, in which domain III of CryIC has been exchanged by domain III of CryIE, is not active against *S. exigua* or *M. brassicae*. Apparently, although the protein is toxic to *M. sexta*, the CryIC sequences running from amino acid

28-462 are not sufficient to kill *S. exigua* and *M. brassicae*. Only when CryIC sequences up to amino acid residue 602 are present in the hybrid (E7) is insecticidal activity against these insects restored.

The present disclosure indicates that amino acid residues from 478-602 of CryIC can confer high insecticidal activity to CryIE against *S. exigua* and *M. brassicae*.

Biotinylation Of Crystal Proteins And Binding Assays

Biotinylation is performed using biotin-N-hydroxysuccinimide ester essentially as described by the manufacturer (Amersham). 1 mg of crystal protein is incubated with 40 ml biotinylation reagent in 50 mM $NaHCO_3$, 150 mM NaCl, pH8, for one hour at 20°C. The solution is loaded on a Sephadex 25 column equilibrated with the same buffer containing 0.1% BSA to remove unbound biotin, and samples of the fractions are spotted on a nitrocellulose membrane. Fractions containing biotinylated crystal proteins are visualized using streptavidine-peroxidase conjugate (Amersham) which catalyzes the oxidation of luminol, resulting in chemiluminescence (ECL, Amersham), and pooled.

Brush border membrane vesicles are isolated as described by Wolfersberger *et al.* (1987) (Comp. Biochem. Physiol. 86a, pp. 301-308) except that the vesicles are washed once more with isolation buffer containing 0.1% Tween 20. Binding of biotinylated crystal proteins to brush border membrane vesicles (100 mg/ml) is performed in 100 ml of PBS containing 1% BSA, 0.1% Tween-20 (pH 7.6). Vesicles (20 µg vesicle protein) are incubated with 10 ng biotinylated crystal proteins in the presence or absence of 1000-fold excess of unlabelled crystal proteins for 1 hour at 20°C. Subsequently, the vesicles are re-isolated by centrifugation for 10 minutes at 14,000 g in an Eppendorf centrifuge, washed twice with binding buffer, re-suspended in sample buffer, denatured by heating, and loaded on 7.5% polyacrylamide gels. After electrophoresis, proteins are blotted to nitrocellulose membranes and biotinylated crystal proteins that are re-isolated with the vesicles are visualized by incubation of the nitrocellulose with streptavidin-peroxidase conjugate (Amersham), which catalyzes the oxidation of luminol, resulting in chemiluminescence (ECL, Amersham).

Because binding to epithelial gut cells is a key step in the mode of action of crystal proteins, the binding of crystal proteins to *S. exigua* brush border membrane vesicles is investigated in heterologous competition experiments. Competition experiments demonstrate that the binding of labeled CryIC (Figure 4A, lane a) and labeled F26 (not shown) can be outcompeted by an excess of both unlabelled CryIC (lane b) or F26 (lane e) but not with an excess of G27 (lane c) or CryIE (lane d). Furthermore, binding of labeled G27 (Figure 4B, lane a) and labeled CryIE (not shown) can be outcompeted by an excess of G27 (lane b) or CryIE (lane d), but not with an excess of CryIC (lane a) or F26 (lane e). From these results, it is concluded that G27 and CryIE recognize the same binding sites on *S. exigua* midgut membranes and that these sites differ from those that are recognized by CryIC and F26. The toxicity and binding assays combined demonstrate that G27 is as toxic as CryIC but that it binds a receptor different therefrom. As insects can develop resistance against a crystal protein by changing receptor binding characteristics, G27 may be used in resistance management programs as an alternative to CryIC.

Expression of *cryIE/cryIC* Hybrid Toxin Genes In Heterologous Systems

The G27 *cryIE/cryIC* hybrid toxin gene is expressed in *E.coli,* and the gene product exhibits at least the same insecticidal activity (at least against *Spodoptera)* as CryIC. Moreover, the product exhibits an increase in such activity when expressed in a *Bacillus thuringiensis* strain (see below). The gene encoding the G27 hybrid toxin is introduced into a suitable shuttle vector system, which is then introduced into an appropriate B.t. host. Such transformed cells are then cultured, and the resulting toxin from both whole cultures and purified crystals is assayed for insecticidal activity.

Construction Of A G27-Containing Shuttle Vector, Transformation Of Bt51, And Purification Of Toxin Protein Therefrom

The gene encoding hybrid G27 (3.4 kb) is cleaved from a pKK233 *E. coli* expression plasmid using *Nco*I and *Xho*I. The *Xho*I site is filled in using the Klenow fragment of *E. coli* DNA Polymerase I. The resulting fragment is ligated to *NcoI/Sma*I-digested pSB635 (pBluescriptKS+, P_{cryIC}, and the CryIA(c) transcription terminator). The resulting plasmid, pSB453, is digested with *Apa*I and *Not*I, yielding a 4.2 kbp fragment carrying the promoter, the hybrid G27 ORF, and the terminator. This fragment is ligated to *Apa*I/*Not*I-digested pSB634 (shuttle vector containing

pBC16.1 and pBluescriptKS+), yielding pSB456 (see Figure 5). Plasmid DNA isolated from *E. coli* DH10B is used to transform the crystal toxin minus B.t. strain, Bt51. Positive isolates are tetracycline resistant, show the presence of pSB456, and contain large inclusions corresponding to a 135 kDa protein (as determined by SDS-PAGE). G27 hybrid toxin samples are prepared from cultures of transformed Bt51 grown through sporulation at 30°C in CYS-Tc[10] media. Insecticidal bioassays (Table 2) are performed on both full whole cultures and on washed crystal protein preparations. Controls include Bt51 (pSB440) containing the CryIC toxin and Bt51 (pSB636) containing CryIE. Toxin concentrations are estimated by SDS-PAGE.

TABLE 2

Toxin	LC_{50}				
	Whole Culture (ppt)		Washed Crystal Protein (ppm)		
CrylC	56(2)	36(2)	40(4)	7.8(2)	8.1(4)
CrylE	79(1)	78(1)	33(4)	11.1(6)	7.5(4)
G27	29(2)	21(2)	25(4)	4.7(4)	6.0(4)
Ratio (IC/G27)	1.93	1.71	1.60	1.66	1.35

Table 2. Bioassay of the hybrid toxin G27 in comparison to CryIC and CryIE. The number of samples is given in parentheses. The hybrid toxin G27 is about 50% more effective than either CryIE or CryIC with respect to toxicity to *Spodoptera sp.*

Production And Selection Of Cry1G/Cry1C Hybrid Toxins

To obtain Cry1G/Cry1C hybrid toxins by *in vivo* recombination, expression vector pHK26 was constructed with a C-terminal truncated *cryIG* (a.k.a. Cry9A) gene (*see,* SEQ ID NO:9) and a N-terminal truncated *cryIC* gene (*see,* SEQ ID NO:1) cloned in tandem. The plasmid pHK26 contains the *trc* promoter followed by bases 1-1650 of *cryIG*, part of the pBluescript SK+ polylinker, and bases 266-3570 of *cryIC*. pHK26 is a derivative of pRM7 in which the cry1A(b) coding sequences from *Nco*I to *Bgl*II have been replaced by part of the *cryIG* gene. The 1650 bp *Nco*I-*Bgl*II *cry1G* fragment was isolated by PCR amplification from plasmid pSB1501 using the primers dGCTAGCCATGGATCAAAATAAACACGGAATTATTG (SEQ ID NO:14) and dCTGGTCAGATCTTTGAAGTAGAGCTCC (SEQ ID NO:15). After allowing

intramolecular recombination of pHK26 in *E. coli* strain JM101, plasmid DNA was isolated and digested with *Bam*HI and *Pin*AI to linearize non-recombinant plasmids. Both *Bam*HI as well as *Pin*AI have unique recognition sites in pHK26, in the polylinker and at position 1074 of *cryIC*, respectively. The overlap between the two truncated *cry* genes in pHK26 that allows recombination extends approximately 1400 base pairs, yet primary interest was in recombinations in or close to domain III. Therefore, *Pin*AI was chosen rather than a second enzyme with a recognition site in the polylinker. This strategy allowed linearization of recombinants with crossovers in front of the *Pin*AI site, thereby effectively selecting for recombinants with crossovers in or near the domain III-encoding sequences.

Digested plasmids were transferred to *E. coli* XL1 cells by transformation, and plasmids from transformants were subsequently analyzed by restriction enzyme digestion and DNA electrophoresis. Over 80% of the transformants contained a plasmid with an insert size corresponding to a single, intact *cry* gene, indicating that selection for homologous recombination events had been efficient. Thirty separate colonies were grown in TB medium and assayed for production of alkaline-soluble protoxins that could be converted to stable 65 kD toxic fragments upon trypsin incubation. This screening method yielded 6 colonies producing a stable 65 kD toxic fragment of the expected size. The location of the crossovers in the hybrid genes was first determined by restriction analysis and finally by nucleotide sequencing. Only three different crossover sites occurred in the 6 hybrid genes thus tested. The hybrid genes were designated HK28-12, HK28-1, and HK28-24. The location of the three different crossover sites is shown in Figures 6A and 6B. The three crossovers are located close to the border between domains II and III, with the three hybrid toxins, designated HK28-12, HK28-1, and HK28-24, differing only one amino acid from each other. Both the solubility of the hybrid protoxins as well as the occurrence of trypsin-resistant products of the expected size suggested that these hybrids proteins were properly folded and might have biological activity. This was subsequently tested against larvae of *Spodoptera exigua*.

Toxicity of CryIG/CryIC Hybrid Toxins Towards *Spodoptera exigua*

The *cryIC*, *cryIG*, and newly isolated *cryIG/cryIC* hybrid genes were introduced in *E. coli* strain XL1-blue and grown for 48 hours at 28°C in TB medium with ampicillin. Cells were disrupted by sonification, and protoxin-containing crystals were isolated by centrifugation. After washing the crystals, the protoxins were solubilized at high pH and the concentration of the 140 kD protoxins in the supernatant was estimated by SDS-PAGE. These samples were assayed for their toxicity to *S. exigua* larvae. Results are shown in Figure 7.

CryIG protoxin is much less toxic to *S. exigua* than CryIC. The hybrids containing domain III of CryIC are significantly more toxic than Cry1G. These results show that, as was demonstrated earlier for CryIE and Cry1A(b), Cry1G can be made considerably more toxic to *S. exigua* by substituting its domain III with that of CryIC. For example, hybrid HK28-24 (SEQ ID NO:12) is much more toxic to *S. exigua* than Cry1G (SEQ ID NO:10). Hybrid HK28-24 is also much more toxic to *S. frugiperda* than Cry1G (data not shown).

Although the present invention has been particularly described with reference to the production of Cry1E/Cry1C and Cry1G/Cry1C hybrid toxins, the routineer in the art will appreciate that many other hybrid toxins having improved insecticidal characteristics may be produced according to the present disclosure. SEQ ID NOs:7 and 8, for example, depict the nucleotide and amino acid sequences, respectively, of a CryIA/CryIC hybrid toxin fragment according to the invention that has improved insecticidal activity. Hybrid toxins may be produced comprising domain III of CryIC and the N-terminal region, including domains I and II, of any other Cry protein. In terms of bioassays, the hybrid toxin-carrying transformants may be grown in SOP media to expedite the assay procedures and reduce the volumes of material required. Moreover, the genes encoding the Cry1E/Cry1C, Cry1G/Cry1C, Cry1A/Cry1C, and/or other hybrid toxins according to the invention may be transferred into toxin-encoding strains of B.t. and/or integrated into the chromosome of selected strains of B.t. or introduced into plant genomes to provide for insecticidal activity *in situ* within the plant *per se*. In this regard, it is particularly preferred that the recombinant DNA encoding the toxins is modified so that codons that are preferred by the plant into which the recombinant DNA is to be inserted are used, whereby expression of the thus

modified DNA in the plant yields substantially similar protein to that obtained by expression of the unmodified recombinant DNA in the organism in which the protein components of the hybrid toxin or toxin fragments are endogenous.

Isolation of Additional B.t. Toxin Genes Based on Sequence Similarity to Known B.t. Toxin Genes

A library is plated at a density of approximately 8,000 pfu per 10 cm Petri dish, and filter lifts of the plaques are made after 7 hours growth at 37°C. The plaque lifts are probed with the cDNA set forth in SEQ ID NO:1, 3, or 9 labeled with 32P-dCTP by the random priming method by means of a PrimeTime kit (International Biotechnologies, Inc., New Haven, CT). Exemplary hybridization conditions are 7% sodium dodecyl sulfate (SDS), 0.5 M $NaPO_4$ pH 7.0, 1 mM EDTA at 50°C. After hybridization overnight, the filters are washed with 2X SSC, 1% SDS at 50°C. Positively hybridizing plaques are detected by autoradiography. After purification to single plaques, cDNA inserts are isolated, and their sequences determined by the chain termination method using dideoxy terminators labeled with fluorescent dyes (Applied Biosystems, Inc., Foster City, CA). This experimental protocol can be used by one of ordinary skill in the art to obtain B.t. toxin genes substantially similar to those set forth in the Sequence Listing.